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Plastid DNA haplotype variation in *Dactylorhiza incarnata* (Orchidaceae): evidence for multiple independent colonization events into Scandinavia

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The early marsh orchid, *Dactylorhiza incarnata* (L.) Soó s. l., grows in medium-rich to rich fens and marshes over much of Europe and parts of Asia. The species is highly polymorphic and different forms may grow together at the same site. In the present study, I tested the hypothesis that these forms represent different migrant populations that have colonized Scandinavia independently of each other, possibly from different source areas. Accessions from Scandinavia and elsewhere were screened for variation at three size-variable plastid marker loci, one polyA repeat, one polyA-polyTA-polyT repeat and one 9 bp indel. Ten haplotypes were defined on basis on the combined variation pattern. The common occurrence of several haplotypes in southern Scandinavia and adjacent areas to the south and the east of the Baltic Sea suggests that *D. incarnata* has been dispersed on repeated occasions across the Baltic. Also, there was some correlation between haplotype composition and morphological form on the island of Gotland, in agreement with the independent colonization hypothesis. Material from northernmost Sweden, Finland and northwest Russia was fixed for a single widespread haplotype, indicating that populations in this area are located farther away from the Pleistocene refugia. *Dactylorhiza incarnata* ssp. *lobelii* from southwest Norway was characterized by a haplotype that was not encountered elsewhere in Scandinavia. Given its proximity to British populations dominated by the same haplotype, it is suggested that *D. incarnata* ssp. *lobelii* was established independently of the other Scandinavian populations, from coastal refugia located in western Europe.

Scandinavia was almost completely covered by ice until the Weichselian ice sheet started to retreat about 15 000 years ago (Björck 1995, Svendsen et al. 2004). Except perhaps for some high alpine species, all plants inhabiting Scandinavia today must have survived the ice age in surrounding ice-free areas and must have migrated to Scandinavia after the ice melted away and opened up new land for colonization (Nordal 1987, Brochmann et al. 2003).

Genetic studies of several deciduous forest trees have revealed that they survived the Weichselian in refugia located far to the south in Europe and that they migrated long distances to reach their present locations in Scandinavia (Hewitt 1996, Taberlet et al. 1998). The typical pattern found in these species is that they contain little genetic variation in Scandinavia, as compared to their postulated refugial areas (Dumolin-Lapègue et al. 1997, King and Ferris 1998, Hampe et al. 2003, Huerz et al. 2004). Although several genetically differentiated refugial areas may be identified in such species, large areas in the north are typically colonized from only a single refugium. Such a pattern is explained by the leading edge hypothesis (Hewitt 1996), which postulates that the northward expansion into previously glaciated areas started from one of the marginal populations of a particular refugium and that this population rapidly expanded its territory and in effect blocked out diaspores arriving later on and which may have carried other genotypes than the already dominant ones.

In contrast to the forest taxa mentioned above, several forest herbs and grasses appear to contain as much genetic diversity in Scandinavia as in surrounding unglaciated areas (Schiemann et al. 2000, Tyler et al. 2002, Borgen and Hultgård 2003). Such species may have survived the Weichselian in refugia located closer to the ice sheet (Stewart and Lister 2001, Provan and Bennett 2008) and may have transmitted a larger fraction of their genetic diversity into Scandinavia than the deciduous forest trees. It is also possible that they reached Scandinvia from different directions as has been described for several Scandinavian mammal species, e.g. brown bear (Taberlet et al. 1995), voles (Jaarola and Tegelström 1995, Jaarola et al. 1999) or some plants including oak (Ferris et al. 1998) and the orchid *Dactylorhiza maculata* ssp. *maculata* (Ståhlberg 2007).

This study describes plastid haplotype variation in *Dactylorhiza incarnata*, a perennial herb growing in fens all over most parts of Scandinavia except for the extreme north. The total distribution ranges from central Asia to the British Isles and from mountain areas in the Mediterranean area to Scandinavia (Hultén and Fries 1986). Today, the species may be encountered in alpine areas close to or even above the timber-line (Kilander 1955, Nilsson 1991, Elven 1994), which indicates that the species could have endured the Weichselian at refugia relatively close to the ice sheet. The species is polymorphic and several distinct forms may be found together within the same locality (Rosvall and Pettersson 1951, Mossberg and Lundqvist 1994). These forms are sometimes treated as separate taxa, which may indicate that each one of them has its own evolutionary history and should have colonized Scandinavia independently of other forms. Although some molecular studies have already questioned whether these forms represent separate lineages (Hedrén et al. 2001), it may still be hypothesised that morphologically polymorphic populations are more genetically diverse than populations consisting of a single form.

The plastid genome in orchids is transmitted by the seed parent (Corriveau and Coleman 1988, Harris and Ingram 1991), whereas the plastids of the pollen parent is not transmitted to the offspring. It follows that the seeds will be fixed for a single haplotype and whenever a new population is established from a single seed, only one haplotype can be found in that population. Presence of multiple haplotypes in a population must either be the result of multiple dispersal events by seeds carrying different haplotypes, or mutations within the population, giving rise to new haplotypes. The former hypothesis would be supported if the same haplotypes are found in potential source areas from which seeds could have been recruited, whereas the latter hypothesis would be supported if the population contains one or several unique haplotypes that are phylogentically close and that are likely to have been derived from an original haplotype within the same population (Lowe et al. 2004).

The aim of this study is to describe recolonization and possible migration routes of *D. incarnata* s. l. into Scandinavia by analysing variation in plastid DNA. It will also be tested whether different morphs of *D. incarnata* represent migrant populations that reached Scandinavia independently, and whether the polymorphism found within sites is correlated to within site diversity as described by plastid haplotype DNA polymorphisms.

Up to now little has been known about the post-glacial migration patterns of *D. incarnata.* Although the species is morphologically variable, it does not express much variation in commonly used marker systems such as allozymes (Hedrén 1996a, 1996b, 2001a, Pedersen 1998), AFLPs (Hedrén et al. 2001) or plastid DNA PCR-RFLPs (Hedrén 2003, Devos et al. 2003), or plastid microsatellites (Pillon et al. 2007). Therefore, a second aim of the study was to find additional markers with the potential to reveal more detailed variation patterns than had been possible before. In a first step, I used universal primers developed from the plastid genome in other angiosperms to sequence potentially variable spacer and intron regions. Whenever potential microsatellite loci or other size variable loci were found in such sequences, I designed *Dactylorhiza*-specific primers to amplify as short portions as possible around these motifs. Each locus was then evaluated in a smaller sample and the most variable ones were selected for further screening.

Material and methods

Plant material

Due to apparent variation in external morphology, the *D. incarnata* (L.) Soó s. l. complex has been subdivided into numerous separate species and infraspecific taxa (Hylander 1966, Nelson 1976, Landwehr 1977, Soó 1980, Bateman and Denholm 1985, Buttler 1991, Delforge 2001, Baumann et al. 2006). Material from Asia is separated as *D. osmanica* (Klinge) Soó and *D. umbrosa* (Kar. & Kir.) Nevski in most floras, but it is clear from previous molecular analyses (Hedrén 2001b, 2003) that they are related to European members of the *D. incarnata* s. l. complex, and hence they are included here as reference material.

In the Scandinavian material, three relatively distinct taxa were kept separate from *D. incarnata* s. s. in the analyses and were used to test the hypothesis of independent immigration. *Dactylorhiza incarnata* ssp. *lobelii* (Verm.) H. Æ. Pedersen is a late-flowering subspecies growing in dune slacks along the west coast of continental Europe from Holland to Denmark, and in similar habitats in southwest Norway. It is similar to *D. incarnata* ssp. *coccinea* (Pugsley) Soó which grows in coastal habitats on the British Isles (Pedersen 2001). *Dactylorhiza incarnata* var. *cruenta* (O. F. Müll.) Hyl. and *D. incarnata* var. *ochroleuca* (Boll) Hyl. have disjunct distributions in Europe and may occur in sympatry with the widespread *D. incarnata* s. s. The first of these varieties is characterized by a heavy spotting on both sides of the leaves, dark purplish flowers, and typically relatively short and flat leaves. It is known from the British Isles, the Alps and the Baltic region. The latter variety is often a tall plant with long, folded leaves and many-flowered inflorescences and is characterized by cream-coloured and yellowish flowers, usually with relatively long and three-lobed lips. It has scattered localities in continental Europe and in the British Isles, but is more common around the Baltic Sea and in Sweden where it is confined to calcareous fens. Several additional taxa have been described from Scandinavia, but they may be regarded as extremes in a more or less clinal variation pattern; examples include the northern *D. incarnata* var. *borealis* (Neuman) Hyl., characterized by its relatively short leaves, and the unusually sturdy, relatively broad-leaved *D. incarnata* var. *latissima* (Zapal.) Hyl. As they are connected by intermediates, their circumscription is more or less arbitrary, and I made no attempt to recognize any of these taxa in the present study. However, the sampled material covers most of the variability present in *D. incarnata* s. l. in Scandinavia, and also much of the variability in the extra-Scandinavian distribution area.

In total, 420 plants of *D. incarnata* s. l. were analyzed (Table 1). The material was taken from the following areas (with numbers of samples in parentheses): Tadjikistan (1), Turkey (43), the Alps (29), Britain (30), countries to the south and southeast of the Baltic Sea (46), Finland and northwest Russia (35), and Scandinavia (236). From Scandinavia, 135 plants were analyzed from the island of

Table 1. Origin (country, region, site) of the *Dactylorhiza incarnata* s. l. material investigated in the present study. Numbers of plants with particular haplotypes are given for each taxon and site. Flora regions in Scandinavia and Finland are abbreviated according to 'Flora Nordica' (Jonsell 2004). Taxa have been abbreviated as follows: inc = *D. incarnata* s. str., cru = *D. incarnata* var. *cruenta*, och = *D. incarnata* var. *ochroleuca*, coc = *D. incarnata* ssp. *coccinea*, lob = *D. incarnata* ssp. *lobelii*, osm = *D. osmanica*, umb = *D. umbrosa*, mad = *D. macedonica*.

Site	Taxa/Haplotypes
Tadjikistan, Pamir, Vallis Jersil	umb 1B
Turkey, Agri, Agri	umb 2A, 2H
Turkey, Tunceli, Pülümür	osm 1D
Turkey, Erzurum, Ascale	osm 3A 3C
Turkey, Erzurum, Erzurum	umb 6C
Turkey, Erzurum, Kop pass	umb 4C
Turkey, Erzurum, Sac pass	umb 1B 2C 2F
Turkey, Erzincan, Kizildag pass	osm 3D 3J
Turkey, Erzincan, Refahiye, 7 km N	osm 2A 2B 2E 1G
Turkey, Erzincan, Refahiye, 20 km E	umb 1D
Turkey, Kars, Ardahan	umb 2F
Turkey, Kars, Kars-Ardahan	umb 1F
Greece, Kilkís, Livádia	mad 10B
Italy, Alto Adige, Grödner Joch	cru 1B
Switzerland, Graubünden, Preda	cru 9A
France, Hautes-Alpes, Les Terasses	cru 1A; inc 17B
Wales, Anglesey, Cors Erddreiniog	coc 1A; inc 9A, 3B
Wales, Anglesey, Rhos-y-Gad	inc 6A
Wales, Anglesey, Newborough Warren	coc 11A
Poland, Pomorze, Prysnievo	inc 2B, 3E
Lithuania, Kurtovénia NP	inc 1B; och 1B
Estonia, Otepää, Kolmjärve	inc 3B
Estonia, Otepää, Murru	inc 1B
Estonia, Virtsu	cru 4B; inc 5B
Estonia, Saaremaa, Kuremäe	inc 5B; och 5B; inc/och 3B
Estonia, Saaremaa, Kuusnõmme	inc 2D, 1E
Estonia, Saaremaa, Köruse	cru 3B, 1D
Estonia, Saaremaa, Muhu	cru 1B
Estonia, Hiiumaa, Körgessaare	inc 3E, 2I
Russia, Karelia, Jansipolje	inc 1B
Russia, Karelia, Kosalma, Uksijärvi	inc 4B
Russia, Karelia, Olonets, Petrosavodsk	inc 3B
Finland, Etelä-Häme, Jyväskylä, Katajaneva	inc 11B
Finland, Oulun Pohjanmaa, Oulu	cru 3B, inc 2B, cru/inc 2B
Finland, Sompion Lappi, Moskuvaara	inc 9B
Sweden, Skåne, Hunneröd	inc 1B
Sweden, Skåne, Lyngsjö	inc 1D
Sweden, Skåne, Måryd	inc 1E
Sweden, Skåne, Åraslövs mosse	och 2B
Sweden, Skåne, Örup	inc 1B
Sweden, Öland, Jordtorp	cru 1B, inc 2B
Sweden, Öland, N Grankärret	inc 1B
Sweden, Öland, Tornrör	inc 1B
Sweden, Öland, Ås	inc 1B
Sweden, Gotland, Agbod	inc 6B, 4E
Sweden, Gotland, Agmyr	cru 1B, inc 2B
Sweden, Gotland, Gylvik	cru 5B, inc 8B, och 1B
Sweden, Gotland, Harudden	inc 3B, 4C, 1D, 2E; och 7B, 3C, 1E
Sweden, Gotland, Hoburgsmyr	cru 2B; inc 3B, 3D; och 2B
Sweden, Gotland I, Hojmyr	och 1B
Sweden, Gotland, Kauparve	cru 1B
Sweden, Gotland, Lillmyr	inc 5B, 5E; och 10B
Sweden, Gotland, Lojsthajd	cru 5B; inc 10B, 1E; och 14B
Sweden, Gotland, Norrlanda	cru 2B; inc 6B; och 2B
Sweden, Gotland, Storsund	cru 6B; inc 2B; och 5B
Sweden, Gotland, Särvät	inc 1E
Sweden, Västergötland, Rådane	inc 2B, och 1B
Sweden, Västergötland, Svenljunga	inc 1B
Sweden, Östergötland, Hagebyhöga	inc 1B, 1E, och 1B, 1E
Sweden, Östergötland, Knäppan	cru 1B, inc 1D; och 1B, 1E
Sweden, Östergötland, Kärna mosse	cru 1B, och 1B
Sweden, Östergötland, L Aska	cru 1B
Sweden, Östergötland, Lagmansro	och 4B
Sweden, Västmanland, Myggkärret	inc 5B
Sweden, Uppland, Kvarnsjön	inc 1B
Sweden, Uppland, Gårdskär	inc 1B
Sweden, Uppland, Långbromossen	inc 1D
Sweden, Dalarna, Gulleråsen	inc 3B
Sweden, Dalarna, Blästmyren	inc 1B
Sweden, Gästrikland, Gustavsmurarna	cru 1B, inc 1B
Sweden, Gästrikland, Körsjön	inc 1D
Sweden, Hälsingland, Loos	inc 3B, 2D
Sweden, Härjedalen, Klinken	cru 1B
Sweden, Jämtland, Blekesjön	cru 1B, inc 2B
Sweden, Jämtland, Lillsjön	cru 1D, inc 1D
Sweden, Jämtland, Enflon	cru 2B, 1D, 1E; inc 1B; och 6B
Sweden, Jmt, Skalberget	inc 2C
Sweden, Jämtland, Vackermyran	cru 1B, 1D; inc 1B, 1D
Sweden, Västerbotten, Bjurträsk	inc 1B
Sweden, Västerbotten, Svansele	cru 3B, inc 5B, och 1B
Sweden, Norrbotten, Suksijoki	cru 3B, inc 1B
Norway, Buskerud, Gjellebekk	inc 5B
Norway, Buskerud, Mjøndalen	inc 4B, 2D
Norway, Rogaland, Sola	lob 2A

Gotland in the Baltic where *D. incarnata* is particularly common and often polymorphic within sites. Six plants were included from the island of Öland, and the remaining 95 plants were taken from the Scandinavian mainland. Exact location of sampling sites can be obtained from the author upon request.

In order to obtain some information on *D. incarnata* haplotypes in southeast Europe, I also included ten plants from one population of *D. macedonica* Hölzinger & Künkele from northern Greece. This taxon is an allotetraploid characterized by the *D. incarnata* s. l. plastid DNA, and to which *D. incarnata* thus must have served as the maternal parent (Hedrén et al. 2007). Haplotype data from this taxon were not included in any calculation of genetic diversity described below.

Molecular methods

DNA was extracted from silica gel-dried flowers (Chase and Hills 1991) or fresh leaf material by the CTAB procedure (Doyle and Doyle 1987), except for the single specimen from central Asia which was extracted from herbarium material.

In order to locate potentially size-variable loci, regions of the plastid DNA for which universal primers were available (Taberlet et al. 1991, Demesure et al. 1995) were sequenced in a set of different members of the genus *Dactylorhiza*. Subsequently, *Dactylorhiza*-specific primer pairs were designed for the amplification of short fragments containing mono- or dinucleotide repeats, or obviously size-variable fragments containing indels or duplications. About 20 such primer pairs were tested on *D. incarnata* s. l. accessions obtained from various parts of the distribution area, and finally three primer pairs were selected for the analysis of the entire material (Table 2). Locus 1 is a polyA mononucleotide repeat located in the *trn*T–*trn*L spacer just upstream *trn*L exon I. Variation at this locus has previously been reported in other orchids, including *Gymnadenia* (Soliva and Widmer 1999), *Cypripedium calceolus* (Fay and Cowan 2001), and *Epipactis* (Squirrell et al. 2002). Locus 3 is an indel located in the *trn*L intron, and some material of *D. incarnata* has a unique deletion at this locus that is not found in any other member of the genus (Hedrén unpubl.). Locus 10b is a polyA–polyTA–polyT repeat located in the *psb*A–*trn*K spacer just upstream of the *trn*K exon I.

In order to exclude the possibility that samples with rare character combinations contained non-*incarnata* plastid genomes (that might have been transferred to *D. incarnata* by means of hybridization with related taxa), an additional locus (6) in the *psb*C–*trn*S pseudospacer (Hedrén 2003), at which *D. incarnata* is fixed for a unique size variant, was also analyzed in some samples. Moreover, samples with rare length variants, or length variants resulting in rare haplotypes, were rerun to confirm that no errors during PCR or data interpretation had occurred.

Sequencing and determination of fragment lengths were performed on an ALFexpress II automated sequencer. Templates for sequencing were obtained by PCR in reaction volumes of 50 µl containing 34 µl ddH$_2$O, 5 µl 10 × reaction buffer buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 4 µl MgCl$_2$ (25 mM), 1 µl dNTPs (10 mM of each nucleotide), 0.4 µl of each primer (25 pmol µl^{-1}), 1.2 µl Taq DNA polymerase (1u µl^{-1}; Roche), and 4 µl template DNA (14 ng µl^{-1}). The PCR products were examined on 1.5% agarose gels, and if single clear bands were seen, the initial PCR products were purified using the Qiagen PCR purification kit. In cases where multiple DNA fragments had been amplified, gel slices were excised and each was purified by means of the Qiagen gel extraction kit. Cycle sequencing reactions were performed by means of the Thermo sequenase Cy5 dye terminator cycle sequencing kit. The sequencing reactions were purified by means of ethanol precipitation. Alignments were straight-forward and performed manually.

Size variable fragments were amplified by means of specific primers and PCR conditions reported in Table 2. All fragments were amplified by an initial round of denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 54.4–57°C (depending on primer pairs; Table 2) for 1 min and extension at 72°C for 60–90 s (depending on expected size), and ended by a final extension at 72°C for 10 min. PCR reactions were performed in a reaction volume of 5 µl containing 4 µl ddH$_2$O, 0.5 µl 10 × reaction buffer buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl$_2$), 0.1 µl dNTPs (10 mM of each nucleotide), 0.0625 µl Cy5-labelled primer (1.5 pmol µl^{-1}), 0.025 µl unlabelled complementary primer (25 pmol µl^{-1}), 0.023 µl AmpliTaq Gold polymerase (5 u µl^{-1}), and 0.4 µl template DNA (14 ng µl^{-1}). The PCR product from each reaction was mixed with 20 µl formamide and appropriate size standards to enable exact size determination of the amplified fragments on the ALFexpress II automated sequencer.

Table 2. Description of plastid indels/microsatellites in *Dactylorhiza incarnata* s. l. examined in the present study. Ann: annealing temperature.

Locus	Type	General region and universal primers	Location	Specific primers for fragment	Sequence 5′–3′	Ann	Approx. fragment size in *D. incarnata* s. l.
1	polyA (Soliva and Widmer 1999)	*trn*L exon 1 – *trn*L exon 2; c/d (Taberlet et al. 1991)	*trn*T – *trn*L intergenic spacer	Cy5trnL5 (=c, Taberlet et al. 1991) trnLR5	CGA AAT CGG TAG ACG CTA CGC CGT TAG AAC AGC TTC CAT TG	57	187–189
3	9 bp deletion	*trn*L exon 1 – *trn*L exon 2; c/d (Taberlet et al. 1991)	*trn*L intron	Cy5trnLR4 trnLF4	ATC CAC TCT CAT TGA AGT TG GAT CTA TCA AAC TCT GGA ATG	55.2	78/87
6	dupl region (Hedrén 2003)	*psb*C – *trn*S; *psb*C/*trn*S (Demesure et al. 1995)	*psb*C – *trn*S pseudospacer	Cy5trnS2 psbC2	AGA GTT TCA GGT CCT ACC TA GTG TTC CTA ACT GCC CAC TT	54.4	370
10b	polyA-TA-T	*trn*H – *trn*K; *trn*H/*trn*K (Demesure et al. 1995)	*psb*A – *trn*K exon 1 intergenic spacer	Cy5trnK1A (= *trn*K, Demesure et al. 1995) HK10F	CCG ACT AGT TCC GGG TTC GA GAA AGG CTT GTT ATT TCA CAG	56	137–145

Data analysis

Haplotypes were distinguished on basis of the combined patterns at the three variable loci (Table 3). Size variants found at the two microsatellite loci were treated as ordered character states according to fragment length (stepwise mutation model; Ohta and Kimura 1973). Haplotypes were connected in a haplotype network by means of Median-joining (Bandelt et al. 1999) using the computer program NETWORK 4.2.0.1 (Fluxus Technology 2007).

Mutations in plastid microsatellite loci should be primarily due to DNA polymerase slippage causing stepwise changes in repeat number, and the ratio of slippage rate to substitution rate has been shown to be higher in the plastid genome than in the nuclear genome of *Arabidopsis thaliana* (Jakobsson et al. 2007). Plastid microsatellites may be capable of revealing the same differentiation patterns as plastid PCR-RFLPs (Deguilloux et al. 2004, but see Doyle et al. 1998). Furthermore, plastid microsatellites may provide more detailed information than plastid PCR-RFLPs (Provan et al. 2001), and plastid microsatellites have been used to reconstruct haplotype networks in e.g. *Abies* under the stepwise mutation model (Clark et al. 2000).

Separate analyses were performed in order to describe large scale differentiation between major partitions of the distribution of *D. incarnata* s. l. and fine scale differentiation patterns in the Scandinavian–Baltic area, respectively.

To describe large scale differentiation patterns the material was grouped into six major regions; (1) Turkey, (2) the Alps, (3) Britain, (4) the area to the southeast of the Baltic Sea, (5) Finland/northwest Russia, and (6) Scandinavia (Sweden and Norway). Pairwise F_{ST} values between regions were calculated in the computer program Arlequin 2.000 (Schneider et al. 2000) with haplotypes treated as unordered characters.

The Arlequin program was also used to perform a nested analysis of molecular variance, AMOVA (Excoffier et al. 1992) in which the genetic variance was partitioned into the between-region, between-sites-within-region, and within-site components. All populations of *D. incarnata* s. l. and all regions, regardless of size, were included in the calculations and haplotypes were treated as unordered characters.

The amount and distribution of haplotype diversity within major regions was estimated by the descriptors total diversity (H_T), mean diversity within sites (H_S) and proportion of total diversity distributed between sites in each region (G_{ST}). Haplotypes were considered as unordered, and calculations were performed in the computer program Haplodiv (Pons and Petit 1995). Regions with less than three sites and sites with less than three individuals were not included in these analyses. Haplotype diversity parameters were also calculated for the combined material, irrespective of region.

Table 3. Definition of haplotypes recognized in *Dactylorhiza incarnata* s. l. For locus 1, numbers of A repeats have been given; for locus 3 presence (1), or absence (0), of 9 bp fragment; and for locus 10b length of microsatellite repeat region in bp. n: numbers of individuals.

Haplotype	Locus 1	Locus 3	Locus 10b	n
A	10	0	38	46
B	10	1	38	283
C	10	1	39	24
D	10	1	40	26
E	10	1	41	28
F	10	1	45	5
G	11	1	38	1
H	11	1	39	2
I	11	1	40	2
J	12	1	39	3

Material from northern Europe (regions 4, 5 and 6 above) was analyzed separately. In these analysis material from Sweden, Norway and Finland was grouped according to flora regions given in Flora Nordica (Jonsell 2004; Table 1), and material from adjoining areas was grouped into regions of comparable size. For each flora region with two or more analyzed individuals, the amount of morphological diversity was quantified by the simple descriptor $1-\Sigma p_{Mi}^2$ (in analogy with the genetic diversity descriptor H, Nei 1973), where p_{Mi} is the frequency of the ith morphological form (taxon) present in the region. Correspondingly, genetic diversity was quantified as $1-\Sigma p_{Hi}^2$, (where p_{Hi} is the frequency of the ith haplotype at the region). A potential general association between the two diversity measures was investigated in the SAS program package, procedure CORR (SAS Inst. 1990).

Differences in haplotype composition between morphologically defined varieties were investigated by means of chi-square tests (SAS program package, procedure FREQ) for certain sites and regions.

Results

Plastid DNA variation

Three size variants were found at locus 1, the polyA microsatellite locus, corresponding to 10, 11, and 12 A repeats, respectively (Table 3). All variants were found in Turkey, whereas most plants from Europe had only 10 repeats. Plants characterized by the deletion in the *trn*L intron, locus 3, were widespread from western Europe to Turkey, but were absent from the areas around the Baltic Sea. The polyA-polyTA-polyT locus, 10b, contained five different length variants with combined repeat regions of 38, 39, 40, 41 and 45 bp, repectively. All size variants were identified in material from Turkey, all but the largest repeat in material from around the southern part of the Baltic Sea, and only the shortest variant from the Alps, Britain and Finland/northwest Russia.

Ten different haplotypes were found when size variants from the three investigated loci were combined (Fig. 1). Haplotype A was the single haplotype characterized by the 9 bp deletion at locus 3. This haplotype is most probably derived from haplotype B, which only differs from A in having the longer fragment at locus 3 (Fig. 1). Haplotypes C, D, E and F differ from B in having fragments at locus 10b that are 1, 2, 3 and 8 bp longer, respectively. Haplotypes G, H and I differ from haplotypes C, D and E, respectively, in having a 1 bp longer fragment (consisting on 11 A repeats) at locus 1. It is not clear whether these haplotypes have evolved in parallel from the haplotypes characterized by 10 A repeats at locus 1, or from each other by mutation at the 10b locus; both possibilities are equally parsimonious. Haplotype J is

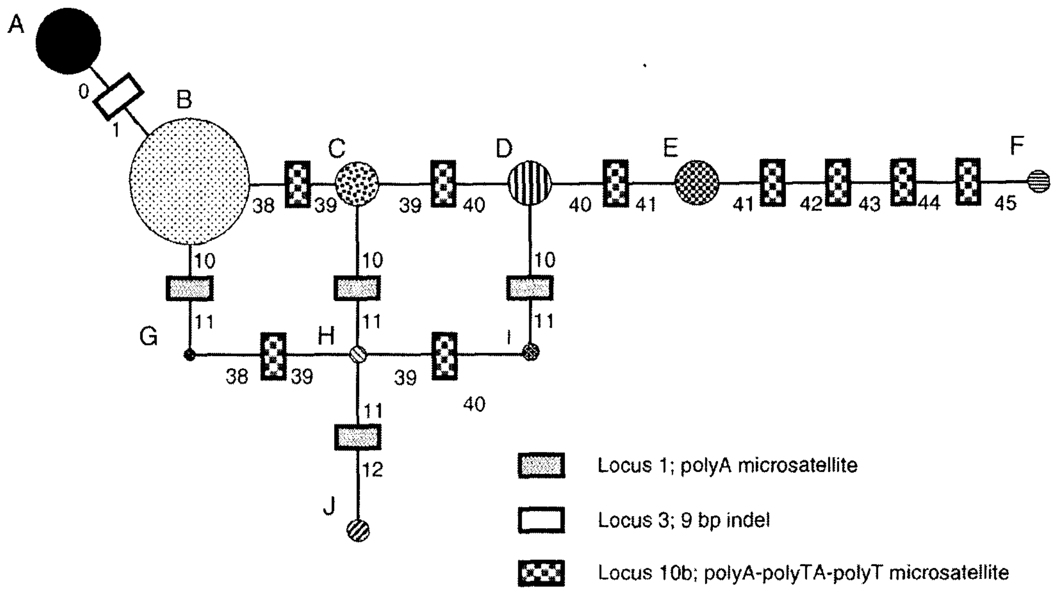

Figure 1. Minimum spanning network connecting haplotypes found in *Dactylorhiza incarnata* s. l. The size of the symbols is roughly proportional to the number of samples in which the particular haplotypes was found (see Table 3 for details).

most closely similar to haplotype H, but differs by an additional A repeat at locus 1.

Geographical patterns

Nine of the ten recognized haplotypes were found in Turkey, which was clearly the most genetically diverse area (Table 4, Fig. 2). Samples from the Alps and from Britain contained haplotypes A and B only, albeit at different frequencies. Haplotype B dominated in northern European samples. Some additional haplotypes were found at low to moderate frequencies in this area. The single population of *D. incarnata* ssp. *lobelii* from western Norway was characterized by haplotype A. Haplotypes D and E were found in many samples from both Scandinavia and from the areas to the southeast of the Baltic Sea. Haplotype I, the only haplotype recorded missing from Turkey, was confined to two specimens sampled on Hiiumaa, Estonia. Haplotype C was encountered in material from both Gotland and the Swedish mainland. Haplotype diversity decreased with latitude in northern Europe, and haplotype B was the only one present in material from Finland, northwest Russia and northernmost Sweden. This haplotype also characterized the single specimen examined from Tadjikistan and a population of the allotetraploid *D. macedonica* from northern Greece (known to be derived from hybridization between *D. incarnata* s. l. and *D. maculata* s. l.; Hedrén et al. 2007).

Genetic diversity patterns

The highest total genetic diversity, H_T, was found in the regional population of Turkey ($H_T = 0.79$; Table 5), followed by the southeast Baltic region, Scandinavia and Britain ($H_T = 0.59$, 0.29, and 0.15, respectively). The Alp region was not considered in these analyses as it contained too few population samples. The mean within-site diversity was also highest in the regional population of Turkey ($H_S = 0.53$). The southeast Baltic region had the highest proportion of genetic diversity distributed between sites ($G_{ST} = 0.45$), followed by the regional populations of Turkey, Scandinavia and Britain ($G_{ST} = 0.33$, 0.15, and 0.09, respectively). As expected, the highest G_{ST} value was found in the joint analysis of all areas such that 54% of the diversity was distributed between sites.

Table 4. Haplotype distribution sorted by taxonomic segregate of *Dactylorhiza incarnata* s. l. and by geographic area.

Region	Taxon	Haplotype									
		A	B	C	D	E	F	G	H	I	J
Tadjikistan	*umbrosa*		1								
Asia Minor	*osmanica*	5	2	3	4	2		1			3
	umbrosa	2	1	12	1		5		2		
Alps	*incarnata*		17								
	cruenta	10	1								
Britain	*incarnata*	15	3								
	coccinea	12									
SE Baltic region	*incarnata*		17		3	7				2	
	cruenta		8		1						
	ochroleuca		6								
	incarnata/ ochroleuca		5								
Gotland	*incarnata*		45	4	4	13					
	cruenta		22								
	ochroleuca		42	3		1					
S Scandinavia	*incarnata*		27		5	2					
	cruenta		4								
	ochroleuca		9			2					
W Norway	*lobelii*	2									
N Scandinavia	*incarnata*		19	2	5						
	cruenta		12		3	1					
	ochroleuca		7								
Finland/ NW Russia	*incarnata*		30								
	cruenta		3								
	incarnata/ cruenta		2								

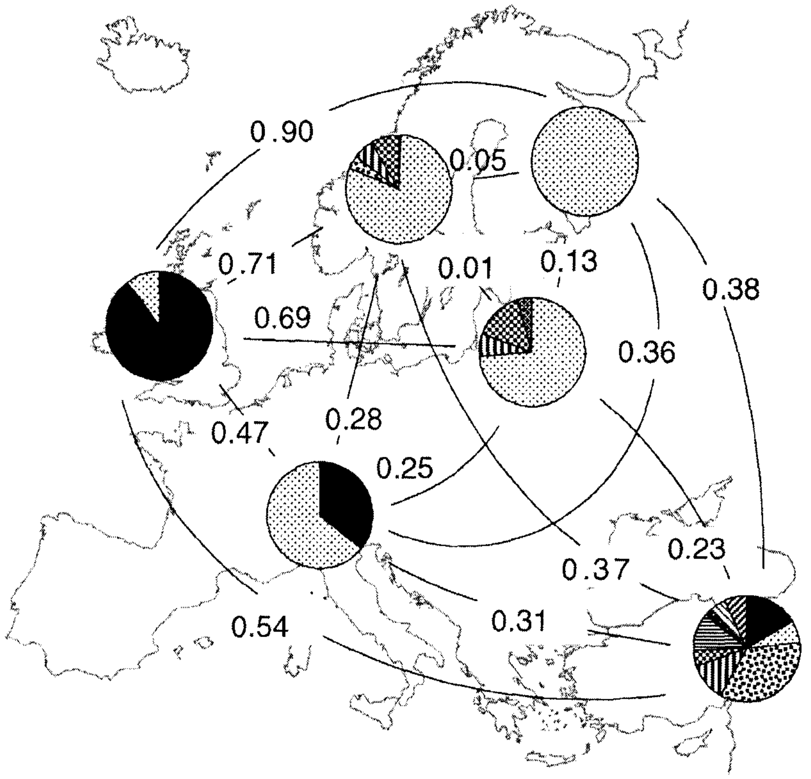

Figure 2. Pairwise F_{ST} values between major regions sampled for *Dactylorhiza incarnata* s. l. All F_{ST} p-values were significant at the 0.05 level, except for the comparison of the southeast Baltic area and Scandinavia, which was non-significant. Distance method: number of different alleles.

The hierarchichal analysis of molecular variance (Table 6) revealed that 35% of the total variation was due to differentiation between major regions, 25% due to differentiation between sites within regions, and the remaining 40% due to differentiation within sites.

Inspection of the pairwise F_{ST} values between major regions (Fig. 2) revealed that the highest values were obtained when the British population was compared with the regional populations from around the Baltic Sea area (Britain vs southeast Baltic area 0.69, Britain vs Finland/ northeast Russia 0.90, and Britain vs Scandinavia 0.71). The lowest values were obtained for the regional populations around the Baltic Sea (F_{ST} ranging from 0.01 to 0.14).

Table 5. Genetic diversity parameters for different parts of the distribution area of *Dactylorhiza incarnata* s. l. Calculations included all populations with more than two analyzed individuals and all major regions with more than two analyzed populations. The region Finland/northwest Russia was omitted as it was fixed for a single haplotype. n_p: numbers of populations; n_i: total number of individuals analyzed within each region; n_a: numbers of haplotypes found within each region. Standard errors are given for each parameter whenever they could be obtained.

Region	n_p	n_i	n_a	H_T	H_S	G_{ST}
Turkey	7	39	9	0.79±0.11	0.53±0.14	0.33±0.11
Britain	3	30	2	0.15±0.13	0.13±0.13	0.09
Baltic	7	42	4	0.59±0.15	0.32±0.12	0.45±0.07
Scandinavia	24	203	4	0.29±0.07	0.25±0.06	0.15
All areas	49	385	10	0.55±0.07	0.25±0.04	0.54±0.07

A moderate but significant general association between morphological diversity and haplotype diversity was found for northern European flora regions (n = 23 flora regions, r = 0.496, p = 0.016).

Comparison of haplotype composition among morphological variants from Gotland revealed significant differences between var. *incarnata* and var. *cruenta* ($\chi^2 = 8.3193$, p = 0.0399), and between var. *incarnata* and var. *ochroleuca* ($\chi^2 = 10.2317$, p = 0.0167), but not between var. *cruenta* and var. *ochroleuca* ($\chi^2 = 1.9876$, p = 0.3702). In contrast, corresponding comparisons within flora regions from mainland Sweden did not reveal any significant differences.

Discussion

Variability at plastid microsatellite loci

The three loci studied differ in the numbers of size variants identified, the two microsatellite loci being more variable than the indel locus. It seems very likely that this pattern is correlated with the mutation rates of these loci and it cannot be excluded that some of the size variants identified, particularly at locus 10b, may have evolved in parallel in different areas. However, the mutation rates at plastid microsatellite loci appear to be very low compared to those commonly reported for nuclear microsatellites (Provan et al. 1999). The 9 bp deletion at locus 3 has not been identified in any related member of *Dactylorhiza* and it is most likely

Table 6. Hierarchic distribution of haplotype diversity in *Dactylorhiza incarnata* s. l. DF = degrees of freedom.

Source of variation	DF	Sum of squares	Variance components	Variation (%)
Between regions	5	37.122	0.12130 Va	34.87
Between sites within regions	77	42.074	0.08777 Vb	25.23
Within sites	333	46.219	0.13880 Vc	39.90
Total	415	125.416	0.34787	

that the deletion is an apomorphy that has evolved within the *D. incarnata* s. l. lineage only once. As the deletion is present in material from the British Isles to eastern Turkey, it must have taken considerable time to disperse over such a large distance in eastern–western direction. Given that this size variant is only associated with a single variant at the two microsatellite loci 1 and 10b (Table 3), the mutation rate at these loci may also be low. It is concluded that the plastid microsatellite loci studied here are suitable for describing phylogeographic patterns in *D. incarnata*, in spite of the fact that some homoplasy was detected in the combined data set.

Two plants from Estonia (Hiiumaa) contained the unique haplotype I which differs from haplotype D by an additional repeat at locus 1, and from haplotype H by a 1 bp longer fragment at locus 10b. It is possible that the haplotype is present also in more southern areas, but has not yet been found due to the restricted sampling from southern sites. Alternatively, it may have arisen in the same general area where it was found in the present study, and if so, it is most plausible that it arose from haplotype D, which is also present in the area to the east of the Baltic Sea.

Colonization of western Norway

The population of *D. incarnata* ssp. *lobelii* from Sola on the Norwegian west coast was the only population from northern Europe that contained the A haplotype (Table 1, 4, Fig. 3). This haplotype was also frequently encountered in material of *D. incarnata* s. l. from the Alps and from Britain, and it is hypothesized that ssp. *lobelii* represents a separate, western colonization route into Scandinavia.

Dactylorhiza incarnata ssp. *lobelii* is also distributed along the west coasts of continental Europe from the Netherlands to western Denmark and may have survived in refugia close to the European Atlantic coast during last glacial maximum. It is closely related to the British *D. incarnata* ssp. *coccinea* and it is possible that they both originate from a common glacial refugium, but diverged from each other as subsets of this refugial population colonized different areas of western and northwestern Europe after the ice withdrawal. The two taxa mainly differ in flower colour, but they both grow in coastal dune slacks (Pedersen 2001) and are similar in general morphology, and *D. incarnata* ssp. *coccinea* is also characterized by a high frequency of the A haplotype (Table 4).

In the traditional phytogeographic classification system, western Norway belongs to the Oceanic flora province, which extends along the western coasts of Europe from Portugal to mid Norway (Sjörs 1956). This region contains many species that do not occur elsewhere in Scandinavia (Hultén 1950), and *D. incarnata* ssp. *lobelii* is obviously only one example of taxa that must have migrated into western Norway from coastal regions of western Europe located further to the south.

Colonization of Scandinavia east of the Scandinavian mountain chain

Scandinavia had haplotypes B, D and E in common with the area to the southeast of the Baltic Sea, and haplotype frequencies were similar between these two areas (Table 4, Fig. 2, 3). Also, all Scandinavian haplotypes were present in the material analyzed from Turkey further to the southeast. In contrast, none of the rare haplotypes encountered Scandinavia, C, D and E, were found in material from Finland or northwestern Russia, or in material from the Alps or from Britain (Fig. 2). The combined pattern suggests that the principal direction of migration into Scandinavia was from the south or the southeast, although it is possible that additional migrants may have come from other directions as well, given the wide distribution of haplotype B in all areas.

In phylogeographic studies of mammal species, it is a common pattern that Scandinavia has been colonized from two directions, from the south as well as from the northeast, as shown by suture zones evident in various molecular data sets in mid Norrland in Sweden (Taberlet et al. 1998, Jaarola et al. 1999). The haplotype distribution pattern found in the present study may be interpreted in a similar fashion; the presence of the single haplotype B in Finland, northwestern Russia and northern Sweden, may be taken as evidence for a northeastern migration route, whereas the common occurrence of the rare haplotypes C, D and E in southern Scandinavia and the areas to the south and southeast of the Baltic Sea may be taken as evidence for a southern migration route. However, haplotype B is also the most frequent haplotype in the south. It follows that the dominance of haplotype B on both sides of the Baltic Sea in the north may equally well be the result of independent losses of rare haplotypes during the northward migration through Scandinavia and through Finland/Russia. If haplotype B dominated in the refugial areas and the other haplotypes were less common, it would be highly probable that the latter should be lost in repeated bottle-necks associated with the northward migration. Accordingly, the common occurrence of haplotype B in northern locations on both sides of the Baltic Sea does not necessarily indicate that these populations are directly connected to each other by historical gene flow.

Diversity patterns

The analysis of molecular variance (Table 6) shows that haplotype diversity in *D. incarnata* is fairly evenly

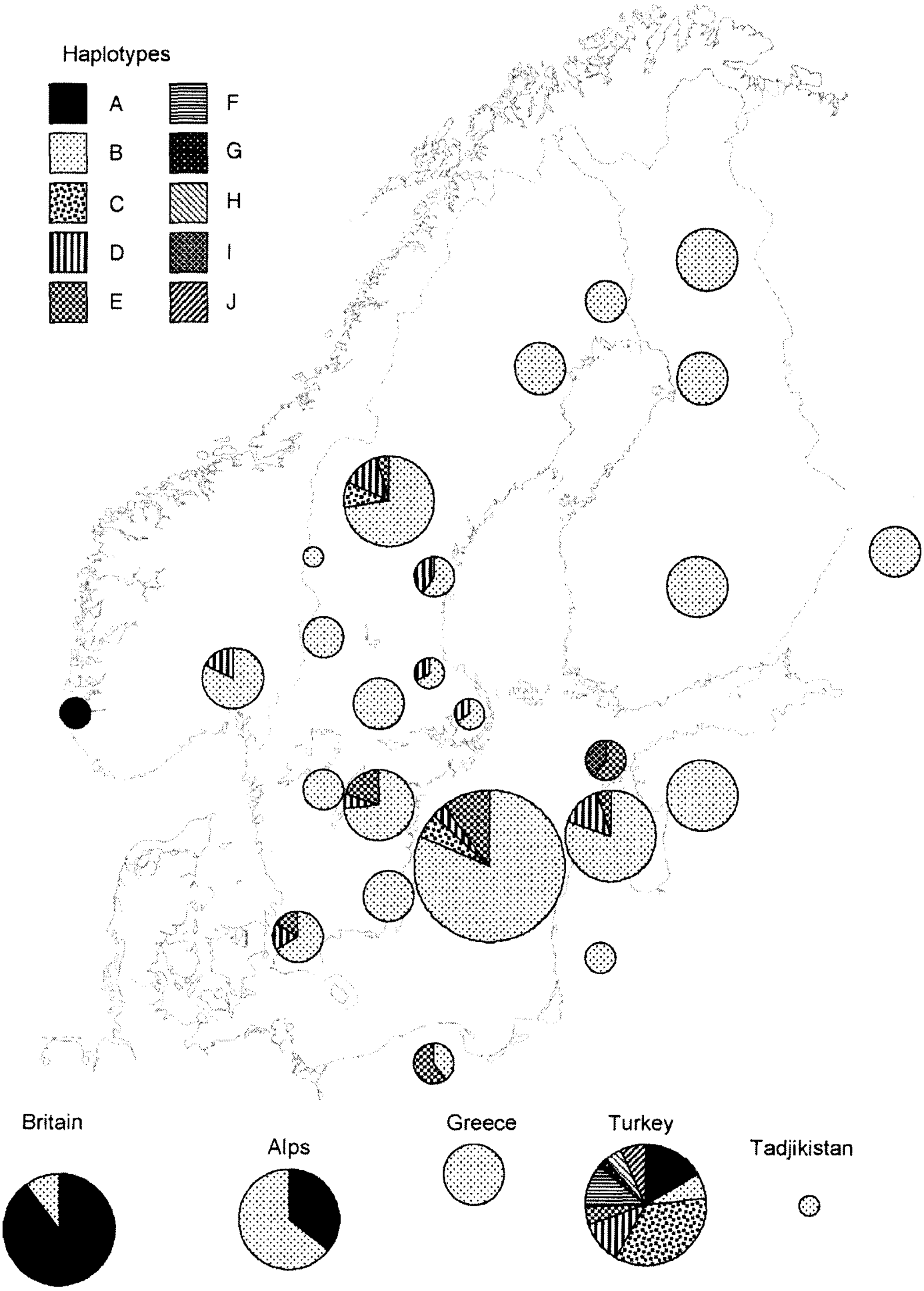

Figure 3. Haplotype frequencies in different flora regions in the Nordic–Baltic area. Flora regions in Scandinavia and Finland are circumscribed as in 'Flora Nordica' (Jonsell 2004) whereas material from adjacent countries are grouped into areas of similar size. The number of analyzed samples in each region is proportional to the area of the corresponding pie diagram, except for the diagram representing Gotland, which is reduced in size compared with the remaining diagrams. Pie diagrams representing Britain, the Alps, Greece, Turkey and Tadjikistan are added for comparison.

distributed at different hierarchic levels; between regions, between sites within regions, and within sites. However, pairwise F_{ST} values presented in Fig. 2 reveal that the regions around the Baltic Sea basin are not particularly differentiated from each other. Accordingly, the main reason for the high degree of differentiation between regions must be due to differentiation of the most geographically distant regions, which were characterized by different frequencies of the most common haplotypes A and B.

Although the total haplotype diversity in Scandinavia ($H_T = 0.29$; Table 5) was lower than that of the area to the southeast of the of the Baltic Sea (0.59), it was still higher than that of Finland/northwestern Russia (0.00) and Britain (0.15). The number of encountered haplotypes was also similar in Scandinavia and in the southeast Baltic area, showing that migration across the Baltic has not seriously affected diversity levels in *D. incarnata*. Orchids are characterized by minute, wind-blown seeds, and they are apparently capable of occasional dispersal over comparably wide water bodies such as the Baltic Sea.

The range of G_{ST} values calculated for the different regional populations, 0.09–0.45, and the G_{ST} value for the entire material, 0.54, are intermediate between those normally found in typically outcrossing and typically inbreeding taxa (Hamrick and Godt 1990, Nybom and Bartish 2000). These values are consistent with observations based on allozyme variation patterns that populations of *D. incarnata* are affected by inbreeding to moderate degrees (Pedersen 1998, Hedrén 2001a).

Correspondence between morphological and haplotype diversity

On the island of Gotland *D. incarnata* var. *cruenta* and var. *ochroleuca* differed significantly from *D. incarnata* var. *incarnata* in haplotype composition (Table 4). Whereas var. *cruenta* was fixed for haplotype B, the other varieties both contained additional haplotypes in moderate frequencies. The pattern may be interpreted such that each variety has colonized Gotland independently on one or several occasions, and that gene flow across varietal borders has not yet homogenized the initial differences in haplotype composition. However, as regards the entire distribution area investigated here, there is considerable overlap between varieties in haplotype composition. No significant differences were found between the varieties on mainland Sweden, possibly indicating a more substantial gene flow between varieties, or that morphologically circumscribed varieties do not represent monophyletic taxa (cf. Hedrén et al. 2001). Yet, the occurrence of several different haplotypes on mainland Sweden still reveal that *D. incarnata* must have colonized the area on multiple occasions.

Material of *D. incarnata* var. *cruenta* from localities in the province of Jämtland in mid Sweden was characterized by the D haplotype. A similar differentiation in var. *cruenta* between this area and southern Sweden was also indicated by variation patterns obtained for a nuclear esterase locus (Hedrén 1996b), and it has also been observed that northern and southern populations of var. *cruenta* are somewhat differentiated in morphology (Nilsson 1991). Still more differentiated from the Scandinavian populations was the material of var. *cruenta* from the Alps, where haplotype A was the dominant haplotype. The overlap in haplotypes between var. *incarnata* and var. *ochroleuca* was also considerable. Taken together these findings suggest that the present subdivision of *D. incarnata* into varieties has little relevance for any phylogeny-based treatment of the complex.

However, the recognition and naming of morphological varieties may still be of importance in a conservation context. A significant correlation was found between morphological and haplotype diversity, and in the process of selecting fen localities for establishment of nature reserves and other protected areas, those harbouring morphologically variable populations of *D. incarnata* s. l. should be of higher interest than those inhabited by morphologically uniform populations.

General conclusions

The present study gives little support to the hypothesis that *D. incarnata* was established in Scandinavia according to the leading-edge model. Several different haplotypes occur in the area today and the diversity in Scandinavia is similar to that of other regions of Europe. *Dactylorhiza incarnata* can be found in the subalpine belt along the Scandinavian mountain chain (Kilander 1955, Elven 1994) as well as in the Alps, and it is ubiquitous in moist depressions on the tree-less Anatolian plateau in Turkey. This distribution indicates that the species was capable of surviving in pockets of better climate that seem to have existed in periglacial areas during the last glacial maximum (cf. Stewart and Lister 2001, Provan and Bennett 2008). If the species was able to survive in refugia close to Scandinavia, the dispersal distance from such refugia should have been relatively short and comparatively little of the genetic diversity that was present in the refugia would have been lost during the recolonization process. Furthermore, it is possible that several refugia may have existed at more-or-less equal distance from the ice margin, and even if each of those was genetically depauperate they may still have been genetically divergent from each other and contributed genetic variants from different directions into Scandinavia and the Baltic area after the ice withdrawal.

Nevertheless, material from northernmost Sweden, Finland and northwestern Russia was fixed for haplotype B, which is consistent with the hypothesis that *D. incarnata* s. l. may have colonized Scandinavia from the south and successively lost genetic variation as it migrated northwards.

The colonization process outlined here for *D. incarnata* may be similar to that described for the birch *Betula pendula* (Palmé et al. 2003). This species may also have survived the last glaciation at relatively high latitudes, and at several sites not very distant from the glaciated areas. Several additional plant species may also have survived in northern refugia located close to the ice margin (Stewart and Lister 2001). This flora may even have included thermophilous trees and shrubs such as oaks, elm and hazel, which grew in sheltered pockets with better microclimate. *Dactylorhiza incarnata* may well have been associated with such a flora.

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References

Bandelt, H.-J. et al. 1999. Median-joining networks for inferring intraspecific phylogenies. – Mol. Biol. Evol. 16: 37–48.

Bateman, R. M. and Denholm, I. 1985. A reappraisal of the British and Irish dactylorchids, 2. The diploid marsh-orchids. – Watsonia 15: 321–355.

Baumann, H. et al. 2006. Orchideen Europas. – Eugen Ulmer.

Björck, S. 1995. A review of the history of the Baltic Sea, 13.0–8.0 ka BP. – Quat. Int. 27: 19–40.

Borgen. L. and Hultgård, U.-M. 2003. *Parnassia palustris:* a genetically diverse species in Scandinavia. – Bot. J. Linn. Soc. 142: 347–372.

Brochmann, C. et al. 2003. Glacial survival or tabula rasa? The history of North Atlantic biota revisited. – Taxon 52: 417–450.

Buttler, K. P. 1991. Field guide to the orchids of Britain and Europe, revised English edition. – Crowood.

Chase, M. W. and Hills, H. G. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. – Taxon 40: 215–220.

Clark, C. M. et al. 2000. Genetic discontinuity revealed by chloroplast microsatellites in eastern North American *Abies* (Pinaceae). – Am. J. Bot. 87: 774–782.

Corriveau, J. L. and Coleman, A. W. 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. – Am. J. Bot. 75: 1443–1458.

Deguilloux, M.-F. et al. 2004. Use of chloroplast microsatellites to differentiate oak populations. – Ann. For. Sci. 61: 825–830.

Delforge, P. 2001. Guide des Orchidées d'Europe, d'Afrique du Nord et du Proche-Orient. – Delachaux et Niestlé.

Demesure, B. et al. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. – Mol. Ecol. 4: 129–131.

Devos, N. et al. 2003. Patterns of chloroplast diversity among western European *Dactylorhiza* species (Orchidaceae). – Plant Syst. Evol. 243: 85–97.

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantity for fresh leaf tissue. – Phytochem. Bull. 19: 11–15.

Doyle, J. J. et al. 1998. Size homoplasy in chloroplast microsatellites of wild perennial relatives of soybean (*Glycine* subgenus *Glycine*). – Mol. Biol. Evol. 15: 215–218.

Dumolin-Lapègue, S. et al. 1997. Phylogeographic structure of white oaks throughout the European continent. – Genetics 146: 1475–1487.

Elven, R. 1994. Norsk flora (6th ed.). – Det Norske Samlaget.

Excoffier, L. et al. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes – application to human mitochondrial DNA restriction data. – Evolution 131: 479–491.

Fay, M. F. and Cowan, R. S. 2001. Plastid microsatellites in *Cypripedium calceolus* (Orchidaceae): genetic fingerprints from herbarium specimens. – Lindleyana 16: 151–156.

Ferris, C. et al. 1998. Chloroplast DNA recognizes three refugial sources of European oaks and suggests independent eastern and western immigrations to Finland. – Heredity 80: 584–593.

Fluxus Technology 2007. Network 4.2.0.1. User Guide. – <http://www.fluxus-technology.com>.

Hampe, A. et al. 2003. Rangewide phylogeography of a bird-dispersed Eurasian shrub: contrasting Mediterranean and temperate glacial refugia. – Mol. Ecol. 12: 3415–3426.

Hamrick, J. L. and Godt, M. J. W. 1990. Allozyme diversity in plant species. – In: Brown, A. H. D. et al. (eds), Plant population genetics, breeding and genetic resources. Sinauer, pp 43–63.

Harris, S. A. and Ingram, R. 1991. Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. – Taxon 40: 393–412.

Hedrén, M. 1996a. Genetic differentiation, polypoidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. – Plant Syst. Evol. 201: 31–55.

Hedrén, M. 1996b. Notes on the esterase variation in Swedish *Dactylorhiza incarnata* s. lat. (Orchidaceae). – Nord. J. Bot. 16: 253–256.

Hedrén, M. 2001a. Conservation priorities in the taxonomically complex genus *Dactylorhiza*. – Lindleyana 16: 17–25.

Hedrén, M. 2001b. Systematics of the *Dactylorhiza euxina/incarnata/maculata* polyploid complex (Orchidaceae) in Turkey: evidence from allozyme data. – Plant Syst. Evol. 229: 23–44.

Hedrén, M. 2003. Plastid DNA variation in the *Dactylorhiza incarnata/maculata* polyploid complex and the origin of allotetraploid *D. sphagnicola*. – Mol. Ecol. 12: 2669–2680.

Hedrén, M. et al. 2001. Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). – Am. J. Bot. 88: 1868–1880.

Hedrén, M. et al. 2007. Patterns of polyploid evolution in Greek marsh orchids (*Dactylorhiza*; Orchidaceae) as revealed by allozymes, AFLPs, and plastid DNA data. – Am. J. Bot. 94: 1205–1218.

Hewitt, G. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. – Biol. J. Linn. Soc. 58: 247–276.

Huerz, M. et al. 2004. Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe. – Mol. Ecol. 13: 3437–3452.

Hultén, E. 1950. Atlas över växternas utbredning i Norden. [Atlas of vascular plant distribution in Norden.] – Generalstabens Litografiska Anstalt.

Hultén, E. and Fries, M. 1986. Atlas of north European vascular plants. – Koeltz.

Hylander, N. 1966. Nordisk kärlväxtflora II. [Flora of Nordic vascular plants]. – Almquist and Wiksell.

Jaarola, M. and Tegelström, H. 1995. Colonization history of north European field voles (*Microtus agrestis*) revealed by mitochondrial DNA. – Mol. Ecol. 4: 299–310.

Jaarola, M. et al. 1999. Colonization history in Fennoscandian rodents. – Biol. J. Linn. Soc. 68: 113–127.

Jakobsson, M. et al. 2007. Evolution of chloroplast mononucleotide microsatellites in *Arabidopsis thaliana*. – Theor. Appl. Genet. 114: 223–235.

Jonsell, B. (ed.) 2004. Flora Nordica. General Volume. – Bergius Foundation.

Kilander, S. 1955. Kärlväxternas övre gränser på fjäll i sydvästra Jämtland samt angränsande delar av Härjedalen och Norge [Upper limits of vascular plants on mountains in southwestern Jämtland and adjacent parts of Härjedalen (Sweden) and Norway]. – Acta Phytogeo. Suec. 35.

King, R. A. and Ferris, C. 1998. Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. – Mol. Ecol. 7: 1151–1161.

Landwehr, J. 1977. Wilde Orchideeën van Europa. – Vereniging tot Behoud van Natuurmonumenten in Nederland.

Lowe, A. et al. 2004. Ecological genetics. – Blackwell.

Mossberg, B. and Lundqvist, Å. 1994. Öländska ängsnycklar. [*Dactylorhiza incarnata* on Öland, Sweden]. – Sv. Bot. Tidskr. 88: 84–96.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. – Proc. Natl Acad. Sci. USA 70: 3321–3323.

Nelson, E. 1976. Monographie und Ikonographie der Orchidaceen-Gattung, III. *Dactylorhiza.* Speich.

Nilsson, Ö. 1991. Nordisk fjällflora (3rd ed.). [Flora of Nordic mountain plants]. – Bonniers.

Nordal, I. 1987. Tabula rasa after all? Botanical evidence for ice-free refugia in Scandinavia reviewed. – J. Biogeogr. 14: 377–388.

Nybom, H. and Bartish, I. V. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. – Persp. Plant Ecol. Evol. Syst. 3: 93–114.

Ohta, T. and Kimura, M. 1973. The model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a genetic population. – Genet. Res. 22, 201 –204.

Palmé, A. E. et al. 2003. Postglacial recolonization and cpDNA variation of silver birch, *Betula pendula.* – Mol. Ecol. 12: 201–212.

Pedersen, H. Æ. 1998. Allozyme variation and genetic integrity of *Dactylorhiza incarnata* (Orchidaceae). – Nord. J. Bot. 18: 15–21.

Pedersen, H. Æ. 2001. Late-flowering dune populations of *Dactylorhiza incarnata* (Orchidaceae): variation patterns and taxonomic inferences. – Nord. J. Bot. 21: 177–186.

Pillon, Y. et al. 2007. Insights into the evolution and biogeography of western European species complexes in *Dactylorhiza* (Orchidaceae). – Taxon 56: 1185–1208.

Pons, O. and Petit, R. J. 1995. Estimation, variance and optimal sampling of gene diversity I. Haploid locus. – Theor. Appl. Genet. 90: 462–470.

Provan, J. and Bennett, K. D. 2008. Phylogeographic insights into cryptic glacial refugia. – Trends Ecol. Evol. 23: 564–571.

Provan, J. et al. 1999. The use of uniparentally inherited simple sequence repeat markers in plant population studies and systematics. – In: Hollingsworth, P. M. et al. (eds), Molecular systematics and plant evolution. Taylor and Francis, pp. 35–50.

Provan, J. et al. 2001. Chloroplast microsatellites: new tools for studies in plant ecology and evolution. – Trends Ecol. Evol. 16: 142–147.

Rosvall, S. and Pettersson, B. 1951. Gotlands orkidéer [The orchids of Gotland, Sweden]. – Bonniers.

SAS Inst. 1990. SAS/STAT User's guide. – SAS Institute.

Schiemann, K. et al. 2000. Allozyme diversity in relation to geographic distribution and population size in *Lathyrus vernus* (L.) Bernh. (Fabaceae). – Plant Syst. Evol. 225: 119–132.

Schneider, S. et al. 2000. Arlequin ver. 2.000: a software for population genetics data analysis. – Genetics and Biometry Laboratory, Univ. of Geneva.

Sjörs, H. 1956. Nordisk växtgeografi [Nordic Plant Geography]. – Scandinavian Univ. Books.

Soliva, M. and Widmer, A. 1999. Genetic and floral divergence among sympatric populations of *Gymnadenia conopsea* s. l. (Orchidaceae) with different flowering phenology. – Int. J. Plant Sci. 160: 897–905.

Soó, R. de 1980. *Dactylorhiza.* – In: Tutin, T. G. et al. (eds), Flora Europea 5. Cambridge Univ. Press, pp. 333–337.

Squirrell, J. et al. 2002. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). – Mol. Ecol. 11: 1957–1964.

Stewart, J. R. and Lister, A. M. 2001. Cryptic northern refugia and the origins of the modern biota. – Trends Ecol. Evol. 16: 608–613.

Ståhlberg, D. 2007. Systematics, phylogeography and polyploid evolution in the *Dactylorhiza maculata* complex (Orchidaceae). Ph D thesis. – Lund Univ., Sweden.

Svendsen, J. I. et al. 2004. Late quaternary ice sheet history of northern Eurasia. – Quat. Sci. Rev. 23: 1229–1271.

Taberlet, P. et al. 1995. Localization of a contact zone beween two highly divergent mitochondrial lineages of the brown bear (*Ursus arctos*) in Scandinavia. – Conserv. Biol. 9: 1253–1261.

Taberlet, P. et al. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. – Plant Mol. Biol. 17: 1105–1109.

Taberlet, P. et al. 1998. Comparative phylogeography and postglacial colonization routes in Europe. – Mol. Ecol. 7: 453–464.

Tyler, T. et al. 2002. Geographic variation and dispersal history in Fennoscandian populations of two forest herbs. – Plant Syst. Evol. 233: 47–64.